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Loss of quin 2 accompanies degranulation of mast cells

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Stimulation of quin 2 loaded mast cells in the presence of 1 mM extracellular calcium produced a rapid and sustained increase in quin 2 fluorescence. This was accompanied by degranulation and the release of histamine. When Ca was replaced by EGTA or when Mn was present, a decrease in fluorescence accompanied degranulation. The increase in quin 2 fluorescence accompanying stimulation of mast cells appears to be due to the interaction of extracellular Ca with quin 2 associated with the secretory granule matrix released upon exocytosis.

Quin 2 *(Mast cell)* *Secretion* *Ca^{2+}*

1. INTRODUCTION

The calcium (Ca)-selective fluorescent chelator quin 2 has been used in various types of cells [1–7] to follow changes in the level of free intracellular Ca, $[Ca^{2+}]_i$. A recent review by Rink and Pozzan [7] describes various aspects of quin 2 uses in cell suspensions. We have used quin 2 in an attempt to measure possible changes in $[Ca^{2+}]_i$ during degranulation of rat peritoneal mast cells and found that a significant portion of the dye is associated with the secretory granule matrix and is released extracellularly upon exocytosis. Our results suggest that a major portion of the increase in fluorescence seen upon stimulation of quin 2 loaded mast cells is due to the association of released quin 2 with extracellular Ca.

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Abbreviations: DMSO, dimethyl sulfoxide; quin 2, methoxyquinoline bis-(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; quin 2/AM, tetraacetoxymethyl ester of quin 2

2. MATERIALS AND METHODS

2.1. *Animals and mast cell isolation and purification*

Mast cells were isolated from the peritoneal and pleural cavities of 150–300 g, male, Sprague-Dawley rats as described [9,10] and purified to >90% homogeneity (as determined by cell counts using a standard hemacytometer at 200× magnification) by centrifugation at 200 × *g* for 10 min at 22°C through 22.5% (w/v) metrizamide (Sigma) in Locke [9]. Purified mast cells were passively sensitized with IgE (rat E myeloma protein IR-162, courtesy of Dr H. Metzger, NIH) by incubation with 12 μg IgE/10^6 cells suspended in Locke. The incubation was for 1 h at 37°C with gentle agitation and done prior to quin 2 loading.

2.2. *Loading mast cells with quin 2*

Purified mast cells (1.5–2.0 × 10^6 cells/ml) were loaded with 10 μM quin 2/AM in Locke. Stocks (10 mM) of quin 2/AM were prepared in DMSO and kept frozen at −20°C. After incubation, an aliquot containing 5 × 10^5 cells was centrifuged (200 × *g* for 5 min), washed once in 1 ml of Locke,

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and resuspended in 2 ml of Locke containing 1 mM $CaCl_2$.

2.3. *Measurement of quin 2 fluorescence*

The quin 2-loaded mast cells were placed in a quartz cuvette and the fluorescence measured at 492 nm with excitation at 339 nm using a Turner model 430 spectrofluorometer. The cell suspension was constantly stirred with a micro-stirring bar and maintained at 37°C by a jacket of circulating water. Fluorescence was continuously recorded on a strip chart recorder. Additions of secretagogues to the cells were usually made without any interruption in the recording.

2.4. *Secretory granule isolation*

Purified populations of mast cells used for granule isolation were incubated for 90 min in the presence of 5 μCi of [^{3}H]quin 2, washed twice with Locke and resuspended in 1 ml of 0.34 M ice-cold sucrose containing 0.1 mM EGTA and 20 mM Hepes. The mast cells were then sonicated for 30 s at 4°C using a Branson sonicator (setting 3) and centrifuged for 5 min at 150 × *g*. The resulting supernatant was centrifuged for 20 min at 4000 × *g* to obtain the granule fraction. Aliquots of resuspended granules, as well as the other fractions, were counted for radioactivity using Aquasol (Dupont-NEN) and a Packard 300 liquid scintillation counter. Histamine measurements were made to confirm granule integrity. Histamine was determined by the fluorometric method of Kremzner and Wilson [11] as described [12]. Aliquots of isolated granule suspension were examined under phase contrast microscopy (× 400) to check for unbroken mast cells. Lactic dehydrogenase (LDH) activity was assayed spectrophotometrically (LDH kit no.500, Sigma).

2.5. *Chemicals*

Compound 48/80 (Burroughs-Welcome), somatostatin (Sigma), and A23187 (Calbiochem) were dissolved as stock solutions (1 mg/ml, 10^{-3} M, and 10 mg/ml, respectively) and kept frozen until use. A23187 was dissolved in DMSO. Quin 2/AM, quin 2 free acid, and [^{3}H]quin 2/AM were purchased from Amersham. Anti-rat IgE serum was purchased from Miles Scientific.

3. RESULTS

3.1. *Incorporation of quin 2/AM into mast cells*

The uptake by mast cells of quin 2/AM and its subsequent hydrolysis to the Ca indicator, quin 2, was followed by the shift in the fluorescence emission spectra from 440 nm – the peak emission for the esterified form of quin 2 – to 480 nm – the peak emission for the free acid (fig.1).

The uptake of quin 2 into mast cells was also followed by incubating mast cells (7×10^5 cells/ml Locke) at 37°C with 10 μM [^{3}H]quin 2/AM (15 mCi/mmol). Aliquots of cells were removed at regular intervals, thoroughly washed with excess, isotope-free buffer, resuspended in scintillation fluid, and counted. As shown in fig.1 (inset) there was a steady uptake of [^{3}H]quin 2/AM which plateaued in 90 min. These results agree with those of Tsien et al. [4], using lymphocytes.

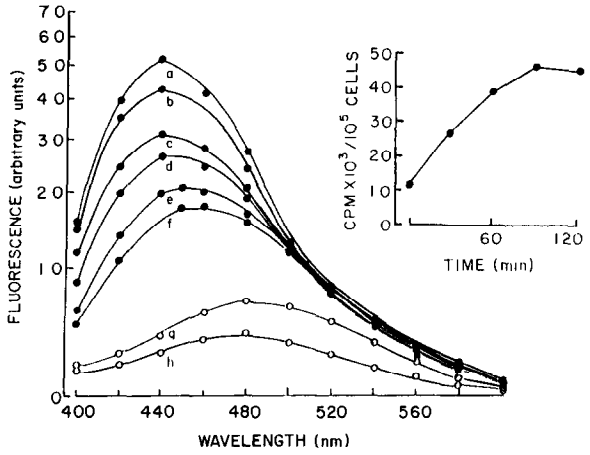

Fig.1. Emission spectra from mast cells incubated for varying periods of time in quin 2/AM showing the shift in the emission peak as the ester form of the indicator is taken up and converted to the free acid form. Excitation was at 339 nm. Curves a–f represent emission spectra measured at particular times after the start of the incubation. Time in min: a, 7; b, 39; c, 78; d, 110; e, 150; f, 174. Curve g is the spectrum obtained after the addition of Triton X-100 and h is the spectrum obtained after the cells were thoroughly washed with buffer. Curves obtained from the loading of 9×10^5 mast cells/2 ml Locke with 10 μM quin 2. Inset: uptake of [^{3}H]quin 2/AM by mast cells over time.

3.2. *Changes in quin 2 fluorescence during stimulation of mast cells in the presence and absence of Ca*

Using the formula and procedures described by Tsien et al. [5] we obtained in 9 separate experiments a value of $[Ca^{2+}]_i$ in unstimulated mast cells of 101 ± 10.9 nM (mean ± SE) which is comparable to that previously reported [3–7]. When quin 2/AM loaded mast cells were stimulated with compound 48/80 (1 μg/ml) or somatostatin (SIRF, 10^{-6} M) in the presence of 1 mM Ca an increased fluorescent signal, such as shown in fig.2, was observed. Fig.2 also shows the $[Ca^{2+}]_i$ levels calculated from the results of this particular experiment. The increase in fluorescence seen in such experiments actually represented 33.3 ± 1.7%, n = 36 of the total possible fluorescence (i.e. the difference between the initial fluorescence before stimulation and the F_{max}, the fluorescence produced after the addition of Triton X-100). Neither 48/80 nor SIRF showed any fluorescence at the wavelengths used.

Mast cells which were washed and suspended in Ca-free Locke containing 0.1 mM EGTA [11,12], showed a marked decrease in the fluorescent signal (fig.3) when stimulated with 48/80 or SIRF but still exhibited a typical degranulation response. This decrease in fluorescence was a consistent finding in 10 separate experiments on different populations of mast cells. However, it was consistently observed only when the quin 2/AM loaded mast cells were bathed for short periods (< 10 min) in Ca-free Locke. The subsequent addition of Ca to the bathing solution produced an increase in fluorescence (fig.3, second arrow).

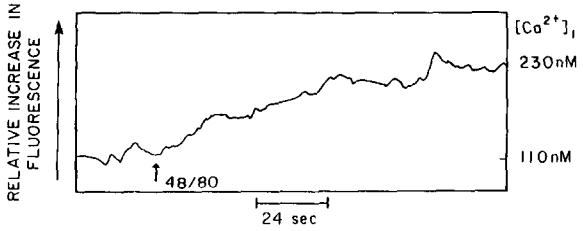

Fig.2. Change in fluorescence from quin 2/AM loaded mast cells following stimulation by compound 48/80 (1 μg/ml) in the presence of Ca.

3.3. *Effect of extracellular manganese on quin 2 fluorescence*

Manganese (Mn) has a greater affinity for quin 2 than does Ca, however its binding to quin 2 does not increase the fluorescence [13]. When Mn (0.1 mM) was added to the solution bathing quin 2 loaded mast cells, an immediate, slight decrease in fluorescence was seen, and when 48/80 was added a typical degranulation (exocytotic) response occurred without any increase in fluorescence. If enough Mn (i.e. 1.0 mM) was present there was a decrease in fluorescence. Raising the extracellular Ca concentration to 2 mM in the presence of Mn (0.1 mM) produced the usual increase in fluorescence. This is in contrast to results from Beaven et al. [14] working with IgE-activated rat basophilic leukemia (RBL) cells where antigen stimulation in the presence of 0.5 mM Mn showed a significant increase in quin 2 fluorescence.

3.4. *Increase of quin 2 fluorescence of the bathing medium upon degranulation*

To test directly for the release of quin 2 during degranulation, we measured the fluorescence of the bathing solution following stimulation of

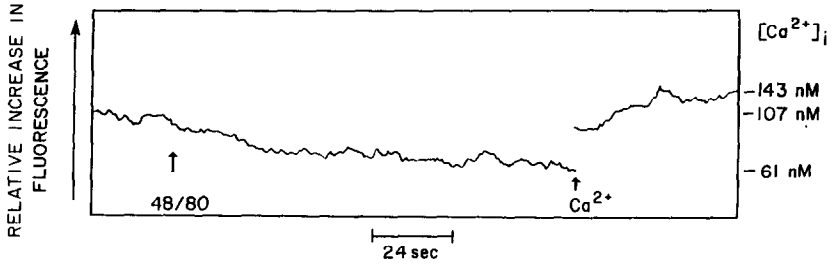

Fig.3. Change in fluorescence from quin 2/AM loaded mast cells bathed in Ca-free Locke following stimulation by compound 48/80 (1 μg/ml), first arrow, and the addition of Ca (1 mM), second arrow. A less than 5 s break in the recording occurred when Ca was added.

quin 2/AM loaded mast cells and compared its fluorescence to that of the bathing fluid obtained from unstimulated cells. Mast cells were separated from the bathing fluid 2 min after the addition of 48/80 and the fluorescence of the supernatant fraction measured before and after quenching by adding Mn (1 mM). The supernatant fluorescence (in arbitrary units) for stimulated mast cells was 9.8 ± 1.0, $n = 11$ and 2.5 ± 0.6, $n = 9$ for non-stimulated cells, an almost 4-fold increase.

3.5. *Release of [^{3}H]quin 2 accompanies degranulation*

To test further for the loss of quin 2 during degranulation, mast cells were loaded with [^{3}H]quin 2/AM (5 μCi) and stimulated with 48/80, SIRF, A23187, or anti-IgE serum or treated with Triton-X. Control cells remained unstimulated. After 5 min at 37°C the cells and supernatant fraction were separated and each sampled for [^{3}H]quin 2 by scintillation counting. As shown in table 1, stimulation by these secretagogues or solubilization by detergent caused an increase in radioactivity in the supernatants. Treatment of degranulated mast cells with 1 M NaCl – a procedure that has been shown to solubilize the released granule matrix thereby freeing it from the exocytotic channels [15] – produced an even greater loss of label (table 1). Mast cells that were incubated in Ca-free Locke containing 2 mM EGTA (a procedure known to inhibit degranulation) [9] did not release [^{3}H]quin 2 (or degranulate) upon stimulation with 48/80.

Table 1

The release of [^{3}H]quin 2 from control and Ca-deprived mast cells before and after stimulation

Treatment	% of total label released
Control mast cells	
Spontaneous release	5.4 ± 0.9 (7)[a]
48/80 (1 μg/ml)	22.9 ± 2.9 (6)
NaCl (1.0 M) after 48/80	35.3 ± 6.4 (3)
A23187 (1.9 nM)	19.1 ± 3.1 (4)
Anti-IgE	15.8 ± 0.7 (4)
Somatostatin (10 μM)	13.6 ± 0.9 (3)
Triton-X (125 μg/ml)	87.1 ± 1.9 (4)
Ca-deprived mast cells	
Spontaneous release	8.4 ± 1.6 (4)
48/80 (1 μg/ml)	9.4 ± 1.4 (4)

[a] Mean ± SE (number of observations)

3.6. *Isolation of secretory granules containing [^{3}H]quin 2*

The release of [^{3}H]quin 2 from mast cells during degranulation suggested to us that a significant portion of the [^{3}H]quin 2 was associated with the secretory granule matrix. To test this notion directly intact membrane bound secretory granules were isolated from unstimulated, [^{3}H]quin 2/AM loaded mast cells and the amount of radioactivity associated with the granules determined. Following sonication of [^{3}H]quin 2 loaded mast cells, intact and partially broken cells were separated from isolated secretory granules by low speed centrifugation (150 × g). The secretory granules in the resulting supernatant fraction were then pelleted by high speed centrifugation (4000 × g). Each fraction (intact and partially broken cells [I], secretory granules [II], and supernatant [III] was then assayed for [^{3}H]quin 2 and histamine. To be certain that this sonication procedure was effective in breaking the mast cells, we assayed fractions II and III for LDH activity. Expressed as a percentage of the total LDH, 7% was associated with the granule fraction (II) whereas 93% was found in the final supernatant fraction (III). To assure that the isolated secretory granules were membrane bounded, they were suspended in hypotonic media and the amount of histamine released into the bathing media determined. As shown in table 2, approx. 1/3 of the total [^{3}H]quin 2/AM that was originally

Table 2

Distribution of [^{3}H]quin 2 and histamine in fractions derived from sonicated mast cells

Fraction	[^{3}H]Quin 2[a] (% total)	Histamine[a] (% total)
Fraction I (unbroken and partially broken cells)	11.8 ± 1.6 (3)	19.9 ± 1.6 (6)
Fraction II (intact secretory granules)	33.3 ± 6.0 (3)	62.9 ± 3.2 (6)
Supernatant	54.4 ± 5.0 (3)	17.2 ± 3.5 (6)

[a] Mean ± SE (number of observations)

incorporated into the mast cell was found in the secretory granule fraction (II). This fraction also contained about 63% of the total histamine.

4. DISCUSSION

We have used quin 2 to measure $[Ca^{2+}]_i$ in stimulated mast cells. Our results show an increase in the fluorescent signal when quin 2 loaded cells were stimulated with 48/80 in the presence of extracellular Ca. However, when Mn was included with the Ca or when extracellular Ca was removed and replaced by EGTA, stimulation of the cells resulted in degranulation and a decrease in the fluorescent signal. Accompanying degranulation was the release of histamine and an increase in the fluorescence of the bathing solution which could be quenched by the addition of Mn. Moreover, degranulation of mast cells loaded with [^{3}H]quin 2 was accompanied by the release of some 20% of the total radioactivity. Solubilization by high salt solution [15] of the released granule matrix trapped within the extracellular exocytotic channels, increased the release of radioactivity to 35% of the total. This amount of quin 2 released to the extracellular fluid can account for the increase in fluorescence that we observed upon stimulation of quin 2 loaded mast cells. In fact the percent increase (i.e. 33%) of the total possible quin-2 fluorescence seen following stimulation by 48/80 is very close to that expected if it was due to the full extrusion of secretory granules as measured by the amount of [^{3}H]quin 2 (i.e. 35%) we found released upon stimulation with 48/80 and extraction with 1.0 M NaCl (table 1). Further when we isolated intact secretory granules from unstimulated mast cells we found that 33% of incorporated [^{3}H]quin 2 (table 2) was associated with the granules. Based on these observations, we conclude that a significant portion of the quin 2 taken up by the mast cell is contained within the secretory granule matrix and as such is released to the extracellular fluid upon degranulation. We suggest that the binding of this released quin 2 to the extracellular Ca accounts for the majority of the increase in quin 2 fluorescence seen upon degranulation of mast cells in the presence of extracellular Ca. When extracellular Ca was removed and EGTA (which removes Ca from quin 2) added or when Mn (which displaces Ca from quin 2) was present there was a decrease in the signal.

The reason quin 2 associates with the granule matrix may simply reflect the fact that secretory granules are the largest intracellular component in mast cells [16] along with the presence in the granules of a variety of esterases [15] capable of hydrolyzing quin 2/AM.

Non-immunologic stimulation of mast cells is known to rely on the mobilization of Ca from cellular pools to initiate exocytosis (degranulation). This cellular Ca can be removed by prolonged chelation [9] and replenished by brief incubation in Ca-Locke (Bibb and Cochrane, unpublished). In the present experiments, we did not observe any increase (or any significant decrease) in the fluorescent signal when quin 2 loaded mast cells were stimulated to degranulate in the absence of extracellular Ca (and without any EGTA present). This suggests to us that either the amount of cellular Ca mobilized in degranulation is too small to be detected by quin 2 or the mobilized Ca is inaccessible to quin 2, because of either location or of a greater affinity for Ca by its normal endogenous binder(s).

The results of our experiments leave unanswered the question of whether $[Ca^{2+}]_i$ increases during non-immunologic stimulation of mast cells. Recent experiments by White et al. [8] have shown increases in quin 2 fluorescence in mast cells stimulated by IgE dependent agents or by 48/80. However, they did not determine the effect of extracellular Mn on the observed fluorescence increase nor did they measure for any loss of quin 2 to the extracellular fluid. Thus it is unclear how much of the signal increase they report is due to the externalization of quin 2. Beaven et al. [14] using RBL cells, which have very few secretory granules [16], did show that significant increases in the quin 2 signal accompanied immunologic stimulation in the presence of Mn indicating little externalization of quin 2 from these cells.

Our results suggest that it may not be appropriate to use quin 2 to follow changes in $[Ca^{2+}]_i$ in cells, like mast cells, where a significant degranulation response accompanies stimulation or secretion.

While this manuscript was in preparation Almers and Neher [17] reported that the calcium fluorescent dye, fura 2, may associate with the

secretory granules of mast cells and may be released upon exocytosis.

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